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Signed R. Mahoney

Dated 12 August 2003

Patents Form 1/77

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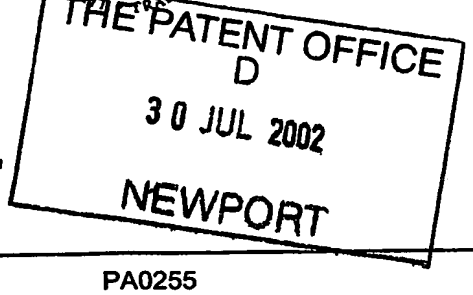

30JUL02 E737054-1 D03022
P01/7700 0.00-0217562.8

Request for grant of a patent

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The Patent Office

Cardiff Road
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1. Your reference — PA0255

2. Patent application number *(The Patent Office will fill in this part)* — 0217562.8 — 30 JUL 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

AMERSHAM BIOSCIENCES UK LIMITED
Amersham Place
Little Chalfont
Buckinghamshire
HP7 9NA

Patents ADP number *(if you know it)* 8371985001

If the applicant is a corporate body, give the country/state of its incorporation — United Kingdom

4. Title of the invention

SITE-SPECIFIC LABELLING OF PROTEINS USING ACRIDONE AND QUINACRIDONE LIFETIME DYES

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

HAMMER, Catriona, MacLeod; ROLLINS, Anthony, John and HAMMETT, Audrey, Grace, Campbell
Amersham plc
The Grove Centre
White Lion Road
Amersham
Buckinghamshire HP7 9LL

Patents ADP number *(if you know it)* 8189375002

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

Yes

Patents Form 1/77

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Claim(s)	6
Abstract	0
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10. If you are also filing any of the following, state how many against each item.

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Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	1
Request for preliminary examination and search *(Patents Form 9/77)*	1
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. I/We request the grant of a patent on the basis of this application.

HAMMER, Catriona, MacLeod

Signature

Date 29 July 2002

12. Name and daytime telephone number of person to contact in the United Kingdom

LIVINGSTONE, Helen
01494 543390

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Site-specific Labelling of Proteins using Acridone and Quinacridone Lifetime Dyes

The present invention relates to reagents and methods for site-specific labelling of proteins with acridone and quinacridone dyes. In particular, the invention relates to new acridone and quinacridone dye derivatives containing thioester activated groups and groups reactive with target molecules containing or derivatised to contain a thioester reactive moiety.

There is an increasing interest in, and demand for, fluorescent labels for use in the labelling and detection of biomolecules. Acridones and quinacridones are highly fluorescent molecules and new acridone and quinacridone dye derivatives having characteristic fluorescence lifetimes have been described for use as labels for target materials. The fluorescence lifetimes of the acridone and quinacridone dyes are generally longer than the lifetimes of other fluorescent labels, as well as naturally occurring fluorescent materials, such as proteins and polynucleotides. The use of fluorescent acridone and quinacridone dyes as fluorescent labels enables easy discrimination from background fluorescence in assays utilising such dyes.

In many applications there is a need to form a permanent link, in the form of a covalent bond, between a fluorescent labelling dye and a target molecule such as a protein. The chemistry of peptide and protein labelling is well documented and a wide range of reagents is now commercially available for the chemical modification of peptides. For a review and examples of protein labelling using fluorescent labelling reagents, see "Non-Radioactive Labelling, a Practical Introduction", Garman, A.J. Academic Press,1997; "Handbook of Fluorescent Probes and Research Chemicals", Haugland, R.P., Molecular Probes Inc., 1992).

Site-specific incorporation of a fluorescent label into a protein or peptide may be of considerable benefit in certain biochemical and biophysical studies, for example fluorescence resonance energy transfer and protein

structure and function studies. One method for the site-specific attachment of reporter groups into a target polypeptide utilises the native chemical ligation reaction. According to this procedure, an unprotected peptide fragment containing an N-terminal cysteine residue and a second reporter-labelled peptide fragment containing an α-thioester group are chemoselectively ligated together at physiological pH, irrespective of their primary sequences, to generate an amide bond at the ligation site. For examples, see reviews by Cotton, G.J. and Muir T.W., Chem. Biol., (1999), 6, R247-260; Giriat, I., Muir, T.W. and Perler, F.B., Genetic Engineering, (2001), 23, 171-199; Muir, T.W., Syn. Lett., (2001), 6, 733-740.

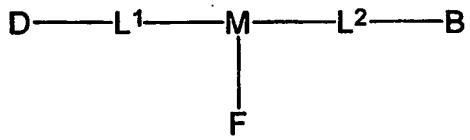
Tolbert, T.J. and Wong, C-H. (Angew. Chem. Int. Ed., (2002), 41, 2171-2174) describe the preparation of fluorescein and biotin thioester derivatives and the reaction of these with N-terminal cysteine-containing recombinant proteins. Schuler, B. and Pannell, L.K. (Bioconjugate Chemistry, 18 July 2002; published on line) reported the preparation of a benzyl thioester of Cy5™ and subsequent reaction with a synthetic polypeptide containing an N-terminal cysteine residue.

However, there are no reports describing thioester derivatives of fluorescent lifetime reporter molecules such as the acridone and quinacridone dyes in which the reporter is also linked covalently to a bioaffinity tag. One of the advantages of these reporters is that their characteristic fluorescence lifetime signatures enables target molecules labelled with such dyes to the differentiated from background fluorescence. Additionally, the acridone and quinacridone classes of dyes may be engineered to have a range of fluorescence lifetimes, distinguishable one from the other, thus enabling applications requiring multiplex detection.

The present invention provides reagents and methods that afford direct attachment of a fluorescent acridone or quinacridone dye to either the N-terminus or C-terminus of a synthetic or recombinant peptide or protein, and

their derivatives, in a site-specific manner, coupled with purification of the resultant labelled molecule.

In a first aspect of the present invention, there is provided a compound of formula (I):



(I)

wherein:

D is a fluorescent dye selected from an acridone and a quinacridone dye;

B is a bioaffinity tag;

F comprises a target bonding group selected from a thioester group and a 1,2-aminothiol group;

M is a group adapted for attaching to F; and

L^1 and L^2 each independently comprise a group containing from 1 – 40 linked atoms selected from carbon atoms which may optionally include one or more groups selected from –NR'–, –O–, –CH=CH–, –CO–NH– and phenylenyl groups, where R' is selected from hydrogen and C_1 – C_4 alkyl.

Suitably, there are 2 to 30 atoms in each of L^1 and L^2, preferably, 6 to 20 atoms.

Preferably, L^1 and L^2 are selected from the group:

$$-\{(CHR')_p-Q-(CHR')_r\}_s-$$

where Q is selected from: –CHR'–, –NR'–, –O–, –CH=CH–, –Ar– and –CO–NH–; R' is hydrogen or C_1 – C_4 alkyl, p is 0 – 5, r is 1 – 5 and s is 1 or 2.

Particularly preferred Q is selected from: –CHR'–, –O– and –CO–NH–, where R' is hereinbefore defined.

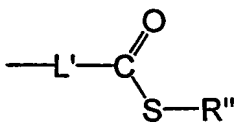
In one embodiment L^2 is a cleavable linker and additionally includes group P which may be suitably selected from a chemically-cleavable group, an enzyme-cleavable group, or a photochemically-cleavable group. Suitable chemically cleavable groups include carbamate esters and carboxylate esters, which are both cleaved under basic conditions. Suitable enzyme cleavable groups may be selected from groups such as ester, amide and phospho-diester groups. Such groups are substrates for, and are hydrolysed by esterases such as proteases and phospho-diesterases. Suitable photocleavable groups P for use in the compound of formula (I) may contain the 4,5-dialkoxy-2-nitrobenzyl alcohol linker (Holmes, C.P., and Jones, D.G., J.Org.Chem., (1995), 60, 2318-2319) or phenacyl linkers (Wang, S., J.Org.Chem., (1976), 41, 3258-3261). These groups undergo efficient photoreaction upon 300nm illumination, resulting in the rapid cleavage of the dye molecule or dye-labelled protein from the bioaffinity tag.

Suitably, the group M may be any suitable functional group adapted for attaching the compound of formula (I) to the target bonding group F. Preferably, M is selected from:

wherein R' is hereinbefore defined.

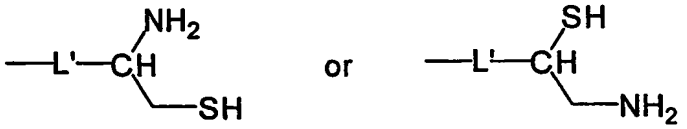
Suitable bioaffinity tags may be selected from biotin, desthiobiotin and metal chelating ligands such as his-tag and iminodiacetic acid, nitrilotriacetic acid and the like. Preferred bioaffinity tags may be selected from biotin and desthiobiotin.

In one embodiment of the present invention, the target bonding group F is a thioester of formula:



wherein L' is a bond or is a group containing from 1 – 30 linked atoms selected from carbon atoms and optionally one or more groups selected from –NH–, –O– and –CO–NH–; and R" is C_1 – C_4 alkyl, C_6 – C_{10} aryl, or C_7 – C_{15} aralkyl, which may be optionally substituted with sulphonate; or is the group $-(CH_2)_2-CONH_2$. In the case where L' is a bond, the target bonding group F is attached directly to group M.

In an alternative embodiment, the target bonding group F is a 1,2-aminothiol group of formula:

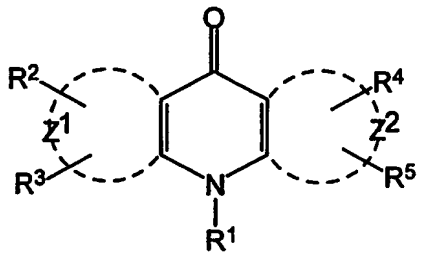


wherein L' is hereinbefore defined.

Thus, the present invention provides fluorescent labelling reagents comprising an acridone or a quinacridone dyes that are modified by incorporating a target bonding group and a bioaffinity tag into the molecule. The target bonding group may be selected from an α-thioester group or a 1,2-aminothiol group, wherein the thioester group is selectively reactive with a 1,2-aminothiol group on a target molecule, suitably a protein or peptide, or a derivative thereof. In the alternative, the acridone or quinacridone dye may contain a 1,2-aminothiol group for reaction with a thioester group on the target. The incorporation of a reactive thioester or, alternatively, a 1,2-aminothiol functionality into the chemical structure of the reporter groups enables the target molecule to be directly labelled in a convenient one step process. According to the methods of the invention, labelling of peptides and proteins is site-specific, irrespective of the composition of the primary sequence. By generating the target primary sequence with either an N-terminal cysteine or an α-thioester functionality, site-specific labelling can be achieved directly, by incubating the target with the appropriate derivative of the acridone or quinacridone dye, the α-thioester and 1,2-aminothiol derivatives respectively. Furthermore, inclusion of a bioaffinity tag in the

labelling reagent allows subsequent purification of the fluorescent dye-labelled protein or peptide.

In one embodiment according to the first aspect, the compound is an acridone dye having the formula (II):



(II)

wherein:

groups R^2 and R^3 are attached to the Z^1 ring structure and groups R^4 and R^5 are attached to the Z^2 ring structure;

Z^1 and Z^2 independently represent the atoms necessary to complete one ring or two fused ring aromatic or heteroaromatic systems, each ring having five or six atoms selected from carbon atoms and optionally no more than two atoms selected from oxygen, nitrogen and sulphur;

at least one of groups R^1, R^2, R^3, R^4 and R^5 is the group F where F is a target bonding group selected from a thioester group and a 1,2-aminothiol group; and

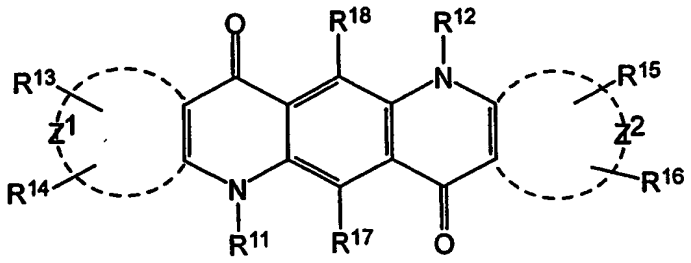
when any of said groups R^1, R^2, R^3, R^4 and R^5 is not said group F, said remaining groups R^2, R^3, R^4 and R^5 are independently selected from hydrogen, halogen, amide, hydroxyl, cyano, amino, mono- or di-$C_1 - C_4$ alkyl-substituted amino, sulphydryl, carbonyl, carboxyl, $C_1 - C_6$ alkoxy, acrylate, vinyl, styryl, aryl, heteroaryl, $C_1 - C_{20}$ alkyl, aralkyl, sulphonate, sulphonic acid, quaternary ammonium and the group $-(CH_2)_n-Y$ and,

when group R^1 is not said group F, it is selected from hydrogen, $C_1 - C_{20}$ alkyl, aralkyl and the group $-(CH_2)_n-Y$;

Y is selected from sulphonate, sulphate, phosphonate, phosphate, quaternary ammonium and carboxyl; and n is an integer from 1 to 6;

provided that at least one of groups R^1, R^2, R^3, R^4 and R^5 is a water solubilising group.

In a second embodiment according to the first aspect, the compound is a quinacridone dye having the formula (III):



(III)

wherein:

groups R^{13} and R^{14} are attached to the Z^1 ring structure and groups R^{15} and R^{16} are attached to the Z^2 ring structure;

Z^1 and Z^2 independently represent the atoms necessary to complete one ring or two fused ring aromatic or heteroaromatic systems, each ring having five or six atoms selected from carbon atoms and optionally no more than two atoms selected from oxygen, nitrogen and sulphur;

at least one of groups R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, R^{16}, R^{17} and R^{18} is the group F where F is a target bonding group selected from a thioester group and a 1,2-aminothiol group; and

when any of said groups R^{13}, R^{14}, R^{15}, R^{16}, R^{17} and R^{18} is not said group F, said remaining groups R^{13}, R^{14}, R^{15}, R^{16}, R^{17} and R^{18} are independently selected from hydrogen, halogen, amide, hydroxyl, cyano, amino, mono- or di-$C_1 - C_4$ alkyl-substituted amino, sulphydryl, carbonyl, carboxyl, $C_1 - C_6$ alkoxy, acrylate, vinyl, styryl, aryl, heteroaryl, $C_1 - C_{20}$ alkyl, aralkyl, sulphonate, sulphonic acid, quaternary ammonium and the group $-(CH_2)_n-Y$; and,

when either of groups R^{11} and R^{12} is not said group F, it is selected from hydrogen, $C_1 - C_{20}$ alkyl, aralkyl and the group $-(CH_2)_n-Y$;

Y is selected from sulphonate, sulphate, phosphonate, phosphate, quaternary ammonium and carboxyl; and n is an integer from 1 to 6;

provided that at least one of groups R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, R^{16}, R^{17} and R^{18} is a water solubilising group.

Suitably, in the compounds according to formula (II) and (III), Z^1 and Z^2 may be selected independently from the group consisting of phenyl, pyridinyl, naphthyl, anthranyl, indenyl, fluorenyl, quinolinyl, indolyl, benzothiophenyl, benzofuranyl and benzimidazolyl moieties. Additional one, or two fused ring systems will be readily apparent to the skilled person. Preferably, Z^1 and Z^2 are selected from the group consisting of phenyl, pyridinyl, naphthyl, quinolinyl and indolyl moieties. Particularly preferred Z^1 and Z^2 are phenyl and naphthyl moieties.

Suitably, at least one of the R groups of the dyes of formula (II) and (III) is a water solubilising group for conferring a hydrophilic characteristic to the compound. Solubilising groups, for example, sulphonate, sulphonic acid and quaternary ammonium, may be attached directly to the aromatic ring structures Z^1 and/or Z^2 of the compound of formula (I) and (II). Alternatively, solubilising groups may be attached by means of a C_1 to C_6 alkyl linker chain to said aromatic ring structures and may be selected from the group $-(CH_2)_n-Y$ where Y is selected from sulphonate, sulphate, phosphonate, phosphate, quaternary ammonium and carboxyl; and n is an integer from 1 to 6. Alternative solubilising groups may be carbohydrate residues, for example, monosaccharides, or polyethylene glycol derivatives. Examples of water solubilising constituents include C_1 - C_6 alkyl sulphonates, such as $-(CH_2)_3-SO_3^-$ and $-(CH_2)_4-SO_3^-$. However, one or more sulphonate or sulphonic acid groups attached directly to the aromatic ring structures of a dye of formula (II) or (III) are particularly preferred. Water solubility may be advantageous when labelling proteins.

In the embodiments according to the first aspect:

i) Aryl is an aromatic substituent containing one or two fused aromatic rings containing 6 to 10 carbon atoms, for example phenyl or naphthyl, the aryl being optionally and independently substituted by one or more

substituents, for example halogen, straight or branched chain alkyl groups containing 1 to 10 carbon atoms, aralkyl and alkoxy for example methoxy, ethoxy, propoxy and n-butoxy;

ii) Heteroaryl is a mono- or bicyclic 5 to10 membered aromatic ring system containing at least one and no more than 3 heteroatoms which may be selected from N, O, and S and is optionally and independently substituted by one or more substituents, for example halogen, straight or branched chain alkyl groups containing 1 to 10 carbon atoms, aralkyl and alkoxy for example methoxy, ethoxy, propoxy and n-butoxy;

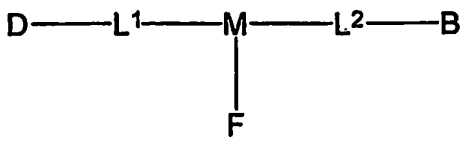
iii) Aralkyl is a $C_1 - C_6$ alkyl group substituted by an aryl or heteroaryl group;

iv) Halogen and halo groups are selected from fluorine, chlorine, bromine and iodine.

By virtue of the reactive group F, the compounds according to the present invention are useful for covalently labelling target biological materials in a site-specific manner for applications in biological detection systems. Suitable target materials include proteins, post-translationally modified proteins, peptides, antibodies, antigens, and protein-nucleic acids (PNAs). The reporter moiety may also be conjugated to species which can direct the path of the reporter within or aid entry to or exit from cells (live or dead); such as for example, long alkyl residues to allow permeation of lipophilic membranes, or intercalating species to localise a reporter in a nucleus or other cellular enclave containing double-stranded DNA.

In a second aspect, there is provided a method for labelling a protein of interest wherein said protein contains or is derivatised to contain an N-terminal cysteine, the method comprising:

i) adding to a liquid containing said protein a compound of formula (I):



(I)

wherein:

D is a fluorescent dye selected from an acridone and a quinacridone dye;

B is a bioaffinity tag;

F comprises a target bonding group selected from a thioester group and a 1,2-aminothiol group;

M is a group adapted for attaching to F; and

L^1 and L^2 each independently comprise a group containing from 1 – 40 linked atoms selected from carbon atoms which may optionally include one or more groups selected from –NR'–, –O–, –CH=CH–, –CO–NH– and phenylenyl groups, where R' is selected from hydrogen and C_1 – C_4 alkyl; and

ii) incubating said compound with said protein under conditions suitable for labelling said protein.

Suitably, there are 2 to 30 atoms in each of L^1 and L^2, preferably, 6 to 20 atoms.

Preferably, L^1 and L^2 are selected from the group:

$$-\{(CHR')_p-Q-(CHR')_r\}_s-$$

where Q is selected from: –CHR'–, –NR'–, –O–, –CH=CH–, –Ar– and –CO–NH–; R' is hydrogen or C_1 – C_4 alkyl, p is 0 – 5, r is 1 – 5 and s is 1 or 2.

Particularly preferred Q is selected from: –CHR'–, –O– and –CO–NH–, where R' is hereinbefore defined.

Covalent labelling using compounds of the present invention may be accomplished with a target having at least one thioester group or 1,2-

aminothiol group as hereinbefore defined. The target may be incubated with an amount of a compound of the present invention having at least one group F as hereinbefore defined that can covalently bind with the complementary group of the target material. The target material and the compound of the present invention are incubated under conditions and for a period of time sufficient to permit the target material to covalently bond to the compound of the present invention. Thus, for example, the thioester group F may be reacted and form a covalent bond with any of the above target materials that contains, or has been derivatised to contain, a 1,2-amino thiol group. These methods and the products resulting from them, for example, reporter-labelled biomolecules are envisaged as further aspects of the invention.

Suitably, the protein of interest may be selected from the group consisting of antibody, antigen, protein, peptide, microbial materials, cells and cell membranes.

In a particular embodiment according to the second aspect, there is provided a method of separating and/or purifying the dye-labelled protein of interest by affinity chromatography utilising the affinity of the bioaffinity tag moiety for an immobilised ligand (or specific binding partner) attached to a support material. Affinity chromatography provides a quick and convenient method to enable the separation of labelled and unlabelled protein molecules under physiological conditions. Proteins labelled with an affinity tag can be selectively bound to an affinity column and any unreacted protein removed by washing the column. Suitable specific binding moieties include avidin or streptavidin (for a biotin tag); immobilised metal ions, for example Cu(II), Ni(II), Fe(II) and Fe(III) (for His-tag or iminodiacetic acid). Methods for affinity purification of proteins will be well known to the skilled person, see for example Ostrove, S, Methods in Enzymology, (1990), Vol 182, page 357.

In a typical labelling procedure, a target peptide or protein containing an N-terminal cysteine residue is agitated with an excess of an acridone or quinacridone dye thioester derivative, for example, 9-oxo-10-{6-oxo-6-[2-

sulfoethyl)thio]hexyl}-9,10-dihydroacridine-2-sulphonic acid (Ace-MESNA) in buffer (typically 200 mM NaCl, 200 mM sodium phosphate) at ~pH 7.3 – 7.4 containing ~1.5% MESNA. The concentration of the target polypeptide in the labelling reaction is generally between 100 μM to 10 mM, whilst the Ace-MESNA is generally present in excess, for example 1.5 to 3-fold molar excess. When the target polypeptide concentration is relatively low, the concentration of Ace-MESNA is usually maintained at or above 1 mM. Generally, for labelling small peptides a solution of Ace-MESNA and MESNA cofactor is directly added to the lyophilised target.

Typically, for site specific labelling of proteins and large polypeptides using the reagents of the present invention, the target is first transferred into an appropriate buffer, which is known not to effect the labelling reaction. An equal volume of a solution of Ace-MESNA and MESNA thiol co-factor in ligation buffer is then added to the protein to give the desired final concentration of the reactants. The reaction mixture is agitated overnight at room temperature. The reaction time may be lowered to less than one hour for high reactant concentrations and, if the stability of the target polypeptide is an issue, the labelling reaction can be performed efficiently at 4°C. On completion of the labelling reaction, dithiothreitol (DTT) is added to a final concentration of ~50 – 250 mM and the desired material isolated by chromatographic procedures.

Various different denaturants, organic solvents and detergents may be added to the reaction buffer when performing native chemical ligation and expressed protein ligation reactions, to aid the ligation of the peptide fragments and/or stabilise the reactants or products. Such reagents may be utilised in the labelling reaction to increase product yield if necessary. Examples include, but are not limited to guanidinium chloride, urea, dimethylformamide, dimethylsulfoxide, acetonitrile, triton X-100, octyl glucoside, 1,6-hexanediol and glycerol.

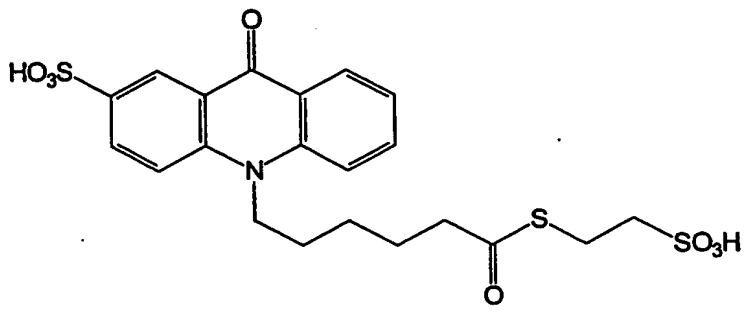
The ligation reaction using the derivatised cyanine dye according to the present invention may be optimally performed at between pH 7.0 and pH 8.0 and at temperatures varying between 4°C and 37°C. It is envisaged that such a range of conditions are compatible to the site-specific labelling reaction described herein.

The advantage of the present method is that it enables the introduction of an extrinsic label into a proteinacious substrate in a regioselective and specific manner, thus minimising any detrimental effects that labelling may have on the biological function of the protein. The importance of controlling stoichiometry of labelling is important where dye overload may interfere with biological activity. In addition, if this controlled labelling stoichiometry is directed towards a single terminal site, rather than towards an internal site, this may have the benefit of further maintaining the biological viability of the labelled species.

The invention is further illustrated by reference to the following examples.

Experimental

1. 9-Oxo-10-{6-oxo-6-[2-sulfoethyl)thio]hexyl}-9,10-dihydroacridine-2-sulphonic acid (Ace-MESNA)



To a stirred solution of 9-oxo-10-{6-carboxyhexyl}-9,10-dihydroacridine-2-sulphonic acid (38.9mg, 0.1mmol) and 2-mercaptoethylsulphonic acid,

sodium salt (MESNA) (25.2mg, 0.153mmol) in anhydrous dimethylformamide (3ml) at ambient temperature was added a solution of dimethylaminopyridine (13.4mg, 0.11mmol) in anhydrous dimethylformamide (1.25ml) followed by a solution of 1-hyroxybenzotriazole (17.9mg, 0.132mmol) in anhydrous dimethylformamide (0.5ml). To this mixture was added as a solid, dried 4A molecular sieves(~1g, <5micron, activated, powder). The mixture was allowed to stir under a dry nitrogen atmosphere for 30 minutes, and this was followed by addition of N,N'-diisopropylcarbodiimide (126mg, 155ul, 1mmol). The mixture was stirred under a dry nitrogen atmosphere for 15 hours. Thin layer chromatography analysis (reverse phase C18 plates, eluent water/acetonitrile (70:30, containing 0.1% TFA) indicated a major UV visible component (rf 0.43) with no trace of starting material (reference rf 0.34). Work up was by filtration through a glass sinter funnel (porosity 3), washing with dimethylformamide (10ml), pouring onto cold diethylether (30ml), and centrifuging (5-10° C, 100rpm, 10 minutes). After trituration with ethylacetate (2x 20ml) and dissolving the dispersed solid into water (25ml), HPLC purification was carried out (Phenomenex Jupiter C18 column, 0-60% gradient elution of water/acetonitrile (containing 0.1% TFA) over 40 minutes. The product was obtained as a yellow solid (28.8mg, 56%), m/z (Maldi) 514, NMR δ_H (300MHz, D_20) 1.45 (2H, m), 1.72 (4H, m), 2.54 (2H, t, CH_2-CO-S), 3.19 (4H, m, SCH_2CH_2), 4.24 (2H, t, NCH_2), 7.23-8.15 (6H, m, aromatic H) and 8.53 (1H, s, 1-CH).

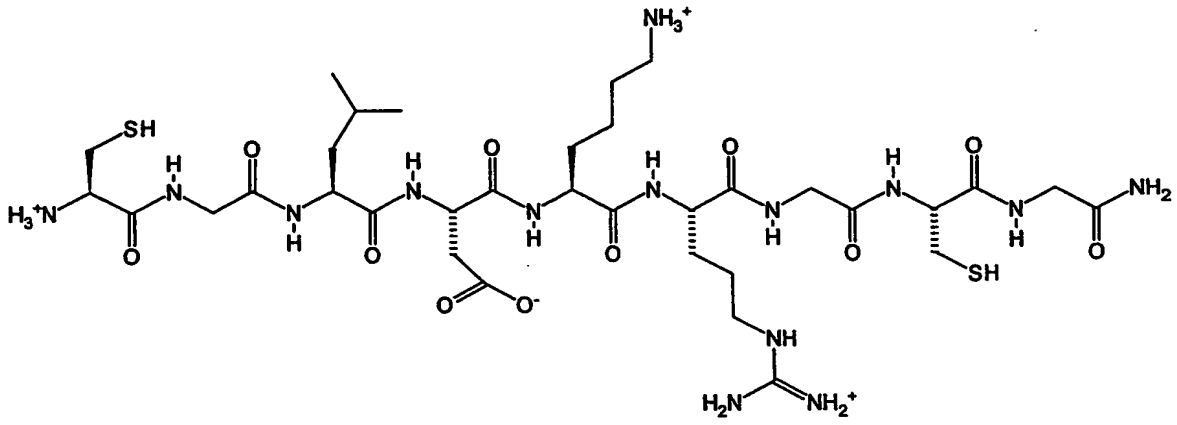
2. Synthesis of Ace-Cys-Gly-Leu-Asp-Lys-Arg -Gly-Cys-Gly-NH_2

2.1 H-Cys(Trt)-Gly-Leu-Asp(OtBu)-Arg(Pmc)-Lys(Boc)Gly-Cys(Trt)Gly-rink amide resin

H-Cys(Trt)-Gly-Leu-Asp(OtBu)-Arg(Pmc)-Lys(Boc)Gly-Cys(Trt)Gly-rink amide resin was synthesised using a commercially available Applied Biosystems Model 433A automated peptide synthesiser using FastMoc™ chemistry, following the instrument manufacturer's recommended procedures throughout. The peptide was synthesised on a 0.25 millimolar scale

employing *O*-(benzotriazol-1-yl)1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) as the activating agent.

2.2 Cys-Gly-Leu-Asp-Lys-Arg -Gly-Cys-Gly-NH_2



H-Cys(Trt)-Gly-Leu-Asp(OtBu)- Lys(Boc)-Arg(Pmc)-Gly-Cys(Trt)Gly-rink amide resin (100mg, theoretical loading 0.36mmol/g) was deprotected and cleaved from solid phase in 95% trifluoroacetic acid (TFA) / 2.5%tri-isopropylsilance (TIS) / 2.5% water (3 mls) at room temperature for 2 hours. The crude product was precipitated into a 10 fold excess of cold diethyl ether, centrifuged at 2500 rpm for 5 minutes and the ether decanted off. The crude peptide was washed twice more with ether and was purified by reverse phase-high performance liquid chromatography (RP-HPLC) [Phenomenex Jupiter C18 column, eluent A: 0.1%TFA/water, eluent B: 0.1%TFA/acetonitrile, gradient : 0-73%B over 30 mins @1ml/min, detection at 214nm]. The product was isolated and lyophilised to afford a colourless fluffy solid (21mg by weight, 60%). Mono-isotopic mass (as carboxylate): 906.09. Found mass (LC-MS): MH+ @ 907.3; M+Na @ 929.6; > 95% pure as judged by RP-HPLC @ 214nm (Phenomenex Jupiter C18 column, eluent A: 0.1%TFA/water, eluent B: 0.1%TFA/acetontrile, 5-50% B over 25mins @ 1ml /min, UV detection at 650nm).

2.3 Ace-Cys-Gly-Leu-Asp-Lys-Arg-Gly-Cys-Gly-NH_2

To solid H-Cys-Gly-Leu-Asp-Lys-Arg-Gly-Cys-Gly-NH_2 (3.2mg by weight, 0.0035mmol) was added a solution of Ace-MESNA (2.5mg, 0.0049mmol, 1.4 equivalents) in 200mM phosphate/200mM NaCl buffer pH 7.2 containing 1.5% 2-mercaptoethanesulphonic acid, sodium salt (500µl). The reaction mixture was stirred for 30 minutes at room temperature in darkness. During incubation, a precipitate formed, which re-dissolved on manual agitation.

A 50µl aliquot of the reaction mix was removed and to this was added 200mM phosphate buffer/200mM NaCl pH 7.2 containing 250mM (final) dithiothreitol (DTT) (100µl). The crude reaction mixture was then analysed by RP-HPLC [250 x 4.6mm Phenomenex Jupiter C4 column, eluent A: 0.1%TFA/water, eluent B: 0.1%TFA/acetonitrile, gradient; 0-50%B over 35 mins at 1ml/min, detection at 220nm and 400nm]. HPLC indicated over 90% consumption of dye (rt. ~14.7min), with major new 400nm-visible peaks at rt. 18.91, 21.60 and 23.42 mins. The remainder of the reaction mix was then worked up by addition of 200mM phosphate buffer/200mM NaCl pH 7.2 containing 250mM (final) dithiothreitol (DTT) (200µl). The crude reaction mixture was then subjected to 3 semi-preparative RP-HPLC runs [250 x 10mm Phenomenex Jupiter C18 column, eluent A: 0.1%TFA/water, eluent B: 0.1%TFA/acetonitrile, gradient; 0-50%B over 40mins at 5ml/min, detection at 220nm and 400nm]. The second of four 400nm-visible peaks (rt. ~26.5mins) was identified as the desired product (MALDI-MS, mono-isotopic mass $C_{53}H_{78}N_{14}O_{17}S_3$ requires 1277, found MH+ 1278). The product from the third HPLC run was isolated and lyophilised as a pale yellow solid (1.4 mg by weight; ~54.5% yield; 97% pure as judged by RP-HPLC at 400nm wavelength. Additional identification was carried out: (LC-MS ES+ single component gives MH+ @ 1278).

2.4 Characterisation of Labelled Peptide

2.4.1 Enzyme Digestion of Ace-Cys-Gly-Leu-Asp-Lys-Arg-Gly-Cys-Gly-NH_2

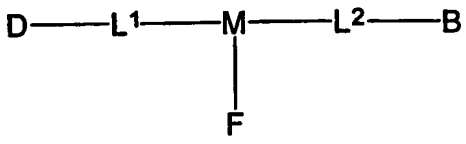
To a solution of Ace-Cys-Gly-Leu-Asp-Lys-Arg-Gly-Cys-Gly-NH_2 (~750μg from the first two preparative HPLC runs from Experimental Section 4.3) in 50mM TRIS buffer pH 7.9 (500μl) containing 0.005% Tween was added Endo Asp-N (2μg) in 50mM TRIS buffer pH 7.9 (100μl). The reaction mixture was stirred overnight at room temperature in the dark under an atmosphere of nitrogen. The reaction mixture was treated with 50mM TRIS buffer pH 7.9 (200μl) containing 250mM (final) dithiothreitol for 30 minutes. The crude reaction mixture was then analysed by RP-HPLC [250 x 4.6mm Phenomenex Jupiter C4 column, eluent A: 0.1%TFA/water, eluent B: 0.1%TFA/acetonitrile, gradient; 0-50%B over 35 mins at 1ml/min, detection at 220nm and 400nm]. HPLC indicated virtual complete enzyme digestion, with a major new 400nm-visible peak at rt. 24.71min and trace of starting substrate at rt. 23.09mins. The crude reaction mixture was then subjected to semi-preparative RP-HPLC [250 x 10mm Phenomenex Jupiter C18 column, eluent A: 0.1%TFA/water, eluent B: 0.1%TFA/acetonitrile, gradient; 0-50%B over 35mins at 5ml/min, detection at 220nm and 400nm]. The main 400nm-visible peak (rt. ~31.18mins) was identified as the desired product (97% by peak area) (MALDI-MS, Ace-Cys-Gly-Leu-OH, mono-isotopic mass $C_{30}H_{37}N_4O_9S_2$ requires 661.78. Found M+H @ 663). In addition, the crude reaction mixture was analysed (LC- MS ES+, diode array detection). Required cleavage product A (Ace-Cys-Gly-Leu) requires 662.79, found MH+ 663. Required cleavage product B (Asp-Lys-Arg-Gly-Cys-Gly-NH_2) requires 634, found MH+ 634. There was no evidence for compound peaks corresponding to non-specific internal Cys residue labelling. The non-cleaved starting material (Ace-Cys-Gly-Leu-Asp-Lys-Arg-Gly-Cys-Gly-NH_2) was also observed as MH_2^{2+} 639.6 (MH+ @1278.2), indicating an endoAsp N enzyme cleavage efficiency of 91.8%.

2.4.2 MS Sequence Analysis of Ace-Cys-Gly-Leu-Asp-Lys-Arg-Gly-Cys-Gly-NH_2

Sequence analysis (MS/MS ion sequence β and γ directions) shows consistency with structure with the terminal Ace-Cys residue observed @ M+ 475.06. No evidence of internal Cys labelling was observed in the sequence.

Claims

1. A compound of formula (I):



(I)

wherein:

D is a fluorescent dye selected from an acridone and a quinacridone dye;

B is a bioaffinity tag;

F comprises a target bonding group selected from a thioester group and a 1,2-aminothiol group;

M is a group adapted for attaching to F; and

L^1 and L^2 each independently comprise a group containing from 1 – 40 linked atoms selected from carbon atoms which may optionally include one or more groups selected from –NR'–, –O–, –CH=CH–, –CO–NH– and phenylenyl groups, where R' is selected from hydrogen and C_1 – C_4 alkyl.

2. A compound according to claim 1 wherein each of L^1 and L^2 contains from 2 to 30 atoms.

3. A compound according to claim 1 wherein each of L^1 and L^2 is selected from the group:

$$-\{(CHR')_p-Q-(CHR')_r\}_s-$$

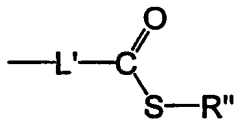
where Q is selected from: –CHR'–, –NR'–, –O–, –CH=CH–, –Ar– and –CO–NH–; R' is hydrogen or C_1 – C_4 alkyl, p is 0 – 5, r is 1 – 5 and s is 1 or 2.

4. A compound according to claim 3 wherein Q is selected from –CHR'–, –O– and –CO–NH–, where R' is hereinbefore defined.

5. A compound according to any of claims 1 to 4 wherein said affinity tag is selected from biotin and desthiobiotin.

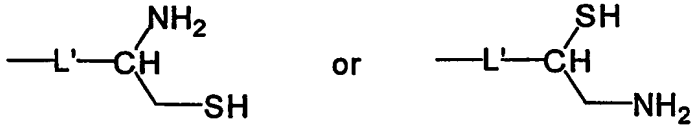
6. A compound according to any of claims 1 to 4 wherein said affinity tag is selected from his-tag, iminodiacetic acid and nitrilotriacetic acid.

7. A compound according to any of claims 1 to 6 wherein the target bonding group F is a thioester of formula:



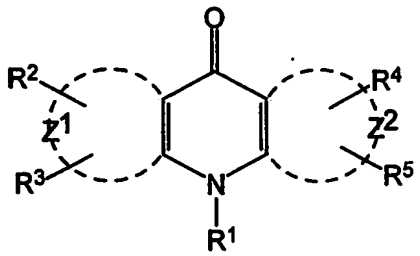
wherein L' is a bond or is a group containing from 1 – 30 linked atoms selected from carbon atoms and optionally one or more groups selected from –NH–, –O– and –CO–NH–; and R" is C_1 – C_4 alkyl, C_6 – C_{10} aryl, or C_7 – C_{15} aralkyl, which may be optionally substituted with sulphonate; or is the group $–(CH_2)_2–CONH_2$.

8. A compound according to any of claims 1 to 6 wherein the target bonding group F is a 1,2-aminothiol group of formula:



wherein L' is hereinbefore defined.

9. A compound according to any of claims 1 to 8 wherein the compound is an acridone dye having the formula (II):



(II)

wherein:

groups R^2 and R^3 are attached to the Z^1 ring structure and groups R^4 and R^5 are attached to the Z^2 ring structure;

Z^1 and Z^2 independently represent the atoms necessary to complete one ring or two fused ring aromatic or heteroaromatic systems, each ring having five or six atoms selected from carbon atoms and optionally no more than two atoms selected from oxygen, nitrogen and sulphur;

at least one of groups R^1, R^2, R^3, R^4 and R^5 is the group F where F is a target bonding group selected from a thioester group and a 1,2-aminothiol group; and

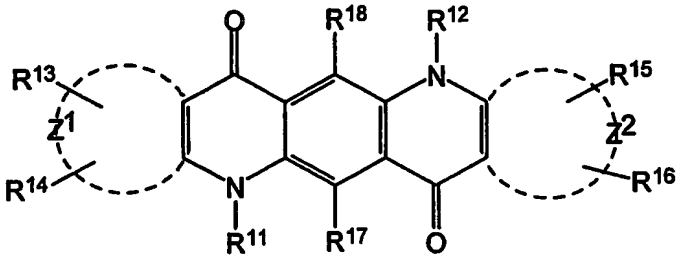
when any of said groups R^1, R^2, R^3, R^4 and R^5 is not said group F, said remaining groups R^2, R^3, R^4 and R^5 are independently selected from hydrogen, halogen, amide, hydroxyl, cyano, amino, mono- or di-$C_1 - C_4$ alkyl-substituted amino, sulphydryl, carbonyl, carboxyl, $C_1 - C_6$ alkoxy, acrylate, vinyl, styryl, aryl, heteroaryl, $C_1 - C_{20}$ alkyl, aralkyl, sulphonate, sulphonic acid, quaternary ammonium and the group $-(CH_2)_n-Y$; and,

when group R^1 is not said group F, it is selected from hydrogen, $C_1 - C_{20}$ alkyl, aralkyl and the group $-(CH_2)_n-Y$;

Y is selected from sulphonate, sulphate, phosphonate, phosphate, quaternary ammonium and carboxyl; and n is an integer from 1 to 6;

provided that at least one of groups R^1, R^2, R^3, R^4 and R^5 is a water solubilising group.

10. A compound according to any of claims 1 to 8 wherein the compound is a quinacridone dye having the formula (III):



(III)

wherein:

groups R^{13} and R^{14} are attached to the Z^1 ring structure and groups R^{15} and R^{16} are attached to the Z^2 ring structure;

Z^1 and Z^2 independently represent the atoms necessary to complete one ring or two fused ring aromatic or heteroaromatic systems, each ring having five or six atoms selected from carbon atoms and optionally no more than two atoms selected from oxygen, nitrogen and sulphur;

at least one of groups R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, R^{16}, R^{17} and R^{18} is the group F where F is a target bonding group selected from a thioester group and a 1,2-aminothiol group; and

when any of said groups R^{13}, R^{14}, R^{15}, R^{16}, R^{17} and R^{18} is not said group F, said remaining groups R^{13}, R^{14}, R^{15}, R^{16}, R^{17} and R^{18} are independently selected from hydrogen, halogen, amide, hydroxyl, cyano, amino, mono- or di-$C_1 - C_4$ alkyl-substituted amino, sulphydryl, carbonyl, carboxyl, $C_1 - C_6$ alkoxy, acrylate, vinyl, styryl, aryl, heteroaryl, $C_1 - C_{20}$ alkyl, aralkyl, sulphonate, sulphonic acid, quaternary ammonium and the group $-(CH_2)_n-Y$; and,

when either of groups R^{11} and R^{12} is not said group F, it is selected from hydrogen, $C_1 - C_{20}$ alkyl, aralkyl and the group $-(CH_2)_n-Y$;

Y is selected from sulphonate, sulphate, phosphonate, phosphate, quaternary ammonium and carboxyl; and n is an integer from 1 to 6;

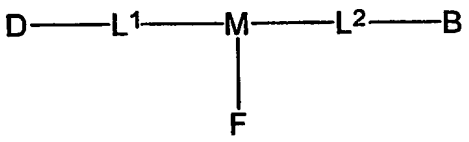
provided that at least one of groups R^{11}, R^{12}, R^{13}, R^{14}, R^{15}, R^{16}, R^{17} and R^{18} is a water solubilising group.

11. A compound according to claim 9 or claim 10 wherein Z^1 and Z^2 are selected independently from the group consisting of phenyl, pyridinyl, naphthyl, quinolinyl and indolyl moieties.

12. A compound according to claim 9 or claim 10 wherein Z^1 and Z^2 are selected from phenyl and naphthyl moieties.

13. A method for labelling a protein of interest wherein said protein contains or is derivatised to contain an N-terminal cysteine, the method comprising:

i) adding to a liquid containing said protein a compound of formula (I):



(I)

wherein:

D is a fluorescent dye selected from an acridone and a quinacridone dye;

B is a bioaffinity tag;

F comprises a target bonding group selected from a thioester group and a 1,2-aminothiol group;

M is a group adapted for attaching to F; and

L^1 and L^2 each independently comprise a group containing from 1 – 40 linked atoms selected from carbon atoms which may optionally include one or more groups selected from –NR'–, –O–, –CH=CH–, –CO–NH– and phenylenyl groups, where R' is selected from hydrogen and C_1 – C_4 alkyl; and

ii) incubating said compound with said protein under conditions suitable for labelling said protein.

14. A compound according to claim 13 wherein each of L^1 and L^2 contains from 2 to 30 atoms.

15. A method according to claim 11 wherein each of L^1 and L^2 is selected from the group:

$$-\{(CHR')_p-Q-(CHR')_r\}_s-$$

where Q is selected from: –CHR'–, –NR'–, –O–, –CH=CH–, –Ar– and –CO–NH–; R' is hydrogen or C_1 – C_4 alkyl, p is 0 – 5, r is 1 – 5 and s is 1 or 2.

16. A method according to claim 15 wherein Q is selected from –CHR'–, –O– and –CO–NH–, where R' is hereinbefore defined.

17. A method according to any of claims 13 to 16 further comprising separating and/or purifying the dye-labelled protein of interest by affinity chromatography.

18. A method according to any of claims 13 to 17 wherein said protein of interest is selected from antibody, antigen, protein, peptide, microbial materials, cells and cell membranes.